

Variation of the Detergent-Binding Capacity and Phospholipid Content of Membrane Proteins When Purified in Different Detergents

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ABSTRACT Purified membrane proteins are ternary complexes consisting of protein, lipid, and detergent. Information about the amounts of detergent and endogenous phospholipid molecules bound to purified membrane proteins is largely lacking. In this systematic study, three model membrane proteins of different oligomeric states were purified in nine different detergents at commonly used concentrations and characterized biochemically and biophysically. Detergent-binding capacities and phospholipid contents of the model proteins were determined and compared. The insights on ternary complexes obtained from the experimental results, when put into a general context, are summarized as follows. 1), The amount of detergent and 2) the amount of endogenous phospholipids bound to purified membrane proteins are dependent on the size of the hydrophobic lipid-accessible protein surface areas and the physicochemical properties of the detergents used. 3), The size of the detergent and lipid belt surrounding the hydrophobic lipid-accessible surface of purified membrane proteins can be tuned by the appropriate choice of detergent. 4), The detergents *n*-nonyl- β -D-glucopyranoside and Cymal-5 have exceptional delipidating effects on ternary complexes. 5), The types of endogenous phospholipids bound to membrane proteins can vary depending on the detergent used for solubilization and purification. 6), Furthermore, we demonstrate that size-exclusion chromatography can be a suitable method for estimating the molecular mass of ternary complexes. The findings presented suggest a strategy to control and tune the numbers of detergent and endogenous phospholipid molecules bound to membrane proteins. These two parameters are potentially important for the successful crystallization of membrane proteins for structure determination by crystallographic approaches.

INTRODUCTION

Studying the function, and in particular the structure, of membrane proteins still remains a challenge. The main reason for this is the amphipathic nature of membrane proteins. They consist of hydrophobic domains, which are embedded in the lipid bilayer membrane, and hydrophilic domains that protrude out of the membrane into the cytosol and extracellular space. For structure determination, membrane proteins are extracted from the biological membrane, purified, and then crystallized. For purification and crystallization, it is imperative that the target membrane protein is stable and monodisperse in the selected detergent (1,2) or detergent mixtures (3,4). Therefore, the choice of the optimal detergent(s) is fundamental and will have an enormous impact on the success of the experimental outcome.

Purified membrane proteins are essentially ternary complexes composed of protein, lipid, and detergent. Here, we address the composition of ternary complexes when purified in detergents of different physicochemical properties. The important role played by detergent in the stabilization and crystallization of ternary complexes has been recognized and addressed in the past (5–8). However, the presence of phospholipids in such complexes has been to some extent

neglected. In this work, we characterized all three components of the ternary complex of purified membrane proteins in a systematic way. Nine nonionic detergents commonly used in membrane protein biochemistry and crystallography were selected to isolate ternary complexes of three model proteins. The model proteins were the urea transporter (UT), from *Desulfovibrio vulgaris*, and the L-arginine/ agmatine antiporter (AdiC) and lactose permease (LacY), from *Escherichia coli*. These membrane proteins were selected based on their different oligomeric states, i.e., trimer (UT), dimer (AdiC), and monomer (LacY), and the availability of their atomic structures (9–11). The UT consists of 10 transmembrane helices and two reentrant helices (9), whereas AdiC and LacY are comprised of 12 transmembrane helices (10,11).

The aim of this study was to characterize the ternary complexes obtained after purification of membrane proteins in different detergents. Knowledge about the amounts of detergent and lipid associated with purified membrane proteins is important for their successful crystallization and subsequent structure determination (12,13). We demonstrate that the size of the detergent and lipid belt surrounding the hydrophobic lipid-accessible surface of purified membrane proteins can be tuned by proper selection of the detergent used for purification. From a sterical point of view, minimization of the detergent/lipid belt volume by the appropriate detergent might allow the formation of crystal contacts and

Submitted June 10, 2013, and accepted for publication February 26, 2014.

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Editor: Robert Nakamoto.

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0006-3495/14/04/1660/11 \$2.00

<http://dx.doi.org/10.1016/j.bpj.2014.02.024>



consequently the growth of membrane protein crystals for x-ray crystallography (12,13).

MATERIALS AND METHODS

Materials

All detergents, i.e., *n*-dodecyl- β -D-maltopyranoside (DDM), *n*-undecyl- β -D-maltopyranoside (UDM), *n*-decyl- β -D-maltopyranoside (DM), *n*-nonyl- β -D-maltopyranoside (NM), *n*-octyl- β -D-maltopyranoside (OM), 7-cyclohexyl-1-heptyl- β -D-maltopyranoside (Cymal-7), 6-cyclohexyl-1-hexyl- β -D-maltopyranoside (Cymal-6), 5-cyclohexyl-1-pentyl- β -D-maltopyranoside (Cymal-5), and *n*-nonyl- β -D-glucopyranoside (NG) were from Affymetrix. UDM and NM were Anagrade, whereas the others were Sol-Grade.

E. coli polar lipid extract (cat. no. 100600C) and *E. coli* total lipid extract (cat. no. 100500C) were from Avanti Polar Lipids (Alabaster, AL). High-performance-liquid-chromatography-grade methanol (CH₃OH) and chloroform (CHCl₃) were from Fisher Scientific (Leicestershire, United Kingdom) and Biosolve (Valkenswaard, The Netherlands), respectively. Acetic acid (glacial) was from Merck KGaA (Darmstadt, Germany). Phosphate-buffered saline (PBS) was purchased from Sigma-Aldrich and prepared according to the manufacturer's protocol by dissolving it in ultrapure water (18.2 M Ω \times cm) obtained from an ELGA Purelab Ultra water system (VWS, Marlow, United Kingdom).

Cloning of UT, AdiC, and LacY

The gene of the urea transporter UT was cloned from genomic DNA of the bacterium *Desulfovibrio vulgaris* strain Hildenborough by polymerase chain reaction (PCR) using the forward primer 5' AAA CAT ATG TTT GGA GAA CAG TTG CTT AAG 3' and the reverse primer 5' AAA GGA TCC TCA GCG AGA CCC GAT CGC GAG 3'. PCR products were digested with the restriction enzymes NdeI and BamHI, and ligated into the pET-15b vector (Novagen, Madison, WI). The pET-15b-UT construct expresses a recombinant UT protein with hexa-His tag at the N-terminus.

The amino acid transporter AdiC from *E. coli* was cloned as described previously (14) into the vector pTrcHisA (Invitrogen, Carlsbad, CA). The pTrcHisA-AdiC construct results in a recombinant AdiC protein with a hexa-His tag at the N-terminus.

The lactose transporter LacY from *E. coli* was cloned into a modified version of the previously described vector pET-21b-rbs-T7-His (15). To generate the vector for the overexpression of LacY, a point mutation was first introduced into the pET-21b-rbs-T7-YdgR-His construct (15) after the STOP codon to generate a SacI restriction site using the forward primer 5' CAC CAC CAC TGA GCT CCG GCT GCT AAC 3' and the reverse primer 5' GTT AGC AGC CGG AGC TCA GTG GTG GTG GTG 3', and the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Next, the construct was digested with HindIII and SacI to remove the *ydgR* gene and its C-terminal extension. The two prehybridized complementary oligonucleotide fragments, which have HindIII and SacI compatible ends, were then inserted into the vector: upper, 5' (HindIII) AGC TTG CGG CCG CAC TCG AGC TGG AAG TTC TGT TCC AGG GGC CCG TCG ACC ATC ACC ACC ATC ATC ACC ATC ACC ACC ACT GAG CT (SacI) 3' and lower, 3' (HindIII) CAG TGG TGG TGA TGG TGA TGA TGG TGG TGA TGG TCG ACG GGC CCC TGG AAC AGA ACT TCC AGC TCG AGT GCG GCC GCA (SacI) 5'. These oligonucleotides introduce a multiple cloning site for the restriction enzymes HindIII, NotI, and XhoI. The *lacY* gene was cloned from genomic *E. coli* strain K12 DNA by PCR using the forward primer 5' AAA AAA GCT TAT GTA CTA TTT AAA AAA CAC 3' and the reverse primer 5' AAA ACT CGA GAG CGA CTT CAT TCA CC 3'. PCR products were digested with the restriction enzymes HindIII and XhoI and ligated into the new vector (pZUDF21-rbs-3C-10His). In this

construct, the target protein has the C-terminal amino acid extension LELEVLFGPVDHHHHHHHHHH, which contains a Prescission (human rhinovirus 3C) protease cleavage site and a deca-His tag. All DNA constructs were verified by sequencing. The calculated molecular masses of the three monomeric recombinant model membrane proteins are ~41.1 kDa (UT), ~50.9 kDa (AdiC), and ~49.2 kDa (LacY).

Overexpression and purification of membrane proteins

For overexpression, pET-15b-UT was transformed into Rosetta 2 (DE3), and pTrcHisA-AdiC and pZUDF21-rbs-LacY-3C-10His into BL21(DE3) pLysS *E. coli* cells. Cultures were grown in Luria Bertani medium supplemented with 0.1 mg/mL ampicillin at 37°C in an orbital shaker. Induction of the protein overexpression was initiated at OD₆₀₀ of 0.5–0.6 with 0.3 mM (UT), 0.3 mM (AdiC), and 0.5 mM (LacY) isopropyl- β -D-thiogalactopyranoside and incubated at 37°C for 3 h. Cells were then harvested by centrifugation (7200 \times g for 25 min at 4°C), resuspended in Lysis buffer (20 mM Tris-HCl, pH 8.0, and 150 mM NaCl) and stored at –20°C. For membrane preparation, frozen cells were thawed and incubated for 5 min with DNase I from bovine pancreas (Sigma-Aldrich, St. Louis, MO; 400 μ g for cells from 12 L of culture) before lysis through a Microfluidizer M-110P (Microfluidics, Newton, MA) at 16,000 psi (four passages). Cell debris were removed by centrifugation (12,000 \times g for 20 min at 4°C) and from the supernatant, membranes were collected by ultracentrifugation (150,000 \times g for 1 h at 4°C). The pellet was homogenized in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM EDTA. An additional ultracentrifugation was performed, the pellet was homogenized in a small volume of Lysis buffer, i.e., 12 mL final total volume, and aliquoted into 2 mL fractions (corresponding to membranes from 2 L of cell culture). Membrane aliquots were stored at –20°C until further use.

For purification, one aliquot of membrane suspension was solubilized for 90 min at 4°C on a rotational shaker in Buffer S (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM betaine, 10% glycerol, and 0.01% (w/v) NaN₃) plus the corresponding concentration of detergent (see Table S1 in the Supporting Material, Solubilization; V_{tot} = 7 mL). After ultracentrifugation (100,000 \times g for 1 h at 4°C), the supernatant was diluted twofold with 5 mM L-histidine in Buffer S and incubated with 0.5 mL preequilibrated Ni-NTA Superflow beads (Qiagen, Valencia, CA) for 2 h at 4°C on a rotational shaker (metal affinity chromatography). The beads were then transferred into a column and washed three times with 5 mL of 5 mM L-histidine in Buffer S plus the corresponding detergent (see Table S1, Washing/Elution/SEC). Finally, the protein was eluted from the beads in the same buffer containing 400 mM imidazole instead of 5 mM L-histidine.

Size-exclusion chromatography of purified membrane proteins

Size-exclusion chromatography (SEC) experiments were performed at 8°C using a Superdex 200 10/300 GL column (GE Healthcare, Wauwatosa, WI) on an Äkta Purifier system (GE Healthcare). The column was calibrated using a commercial high-molecular-weight gel filtration calibration kit (GE Healthcare). Marker proteins were thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa). The column was equilibrated with 1.5 column volumes of 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl containing the corresponding detergent (SEC buffer; see Table S1 for detergent concentrations). Before injection, purified protein samples were centrifuged at 100,000 \times g for 30 min at 4°C. The sample was injected into the fast-protein liquid chromatography system using a 200 μ L loading loop. The flow rate was 0.5 mL/min during SEC. Peak fractions were collected and stored at 4°C for subsequent protein, detergent, and phospholipid determinations. Protein concentrations ranged from 0.1 to 0.4 mg/mL. It is important to point out that to avoid co-concentration of detergent, SEC peak fractions were never concentrated.

Determination of the membrane protein content in SEC peak fractions

The bicinchoninic acid assay (Pierce, Thermo Scientific, Waltham, MA) was used for protein determination because of its compatibility with detergents, low variability, and high sensitivity (16).

Determination of the detergent content in SEC peak fractions

The detergent content in SEC peak fractions of purified membrane proteins was determined by measuring the diameters of detergent-containing drops on a hydrophobic surface similar to Engel et al. (17) and Kaufmann et al. (18). This method is based on the drop diameter as a function of the surface tension (detergent content). Briefly, standard solutions at concentrations below the critical micelle concentration (CMC) of the corresponding detergent were prepared for, e.g., UDM: 0.1, 0.2, 0.3, 0.5, and $0.6 \times \text{CMC}_{\text{UDM}}$. From these solutions, 20 μL drops were deposited on a flattened-parafilm surface together with the samples from SEC. Before deposition, test samples were diluted in SEC buffer (without detergent) below the CMC of the corresponding detergent (10–100 times, depending on the CMC) and incubated overnight at 18°C. Dilution below the CMC dissolves the micelles into detergent monomers, thus avoiding possible disturbing effects (18). Images of the drops were recorded from above with a digital camera, and the diameters of the drops were measured in pixels using the image processing software Adobe Photoshop. To determine the amount of detergent in SEC peak fractions, standard curves were generated by plotting drop diameters versus \log_{10} of the concentrations of the standard solutions. The amount of detergent bound to membrane proteins was calculated by subtracting the amount of detergent in the corresponding SEC buffer from the total amount of detergent in the SEC peak fraction (which also contains membrane proteins). Detergent concentrations in SEC peak fractions of the studied membrane proteins ranged from 1 (DDM) to 34 mM (OM).

Determination of the phospholipid content in SEC peak fractions

The total amount of phosphorus, and thus of phospholipids, in samples of purified membrane proteins was determined spectrophotometrically as described on the Avanti Polar Lipids web site (www.avantilipids.com; see Determination of Total Phosphorus). This protocol is based on Chen et al. (19), and Fiske and Subbarow (20). From SEC peak fractions of membrane proteins purified in different detergents, 100–200 μL samples were pipetted into 16 \times 125 mm disposable glass tubes with screw cap closures. In a similar way, different amounts of phosphorus standard solution (cat no. P3869, Sigma-Aldrich; $[\text{P}] = 0.65 \text{ mM}$) were placed into six separate tubes, i.e., 0 μL (0 μmol), 50 μL (0.0325 μmol), 100 μL (0.065 μmol), 125 μL (0.08125 μmol), 250 μL (0.1625 μmol), and 350 μL (0.2275 μmol) to generate a calibration curve. To each standard tube, 100–200 μL (depending on the detergent) of the corresponding SEC buffer was also added. To decompose the organic sample and produce inorganic phosphate, 450 μL of 8.9 N H_2SO_4 solution was added to each tube and heated in a heating block in a hood at 200–225°C for 30 min. Tubes were then removed from the block and allowed to cool before addition to each tube of 150 μL of 30% (w/w) H_2O_2 (cat no. 216763, Sigma-Aldrich) and continued heating for an additional 30 min. After this step, if any brown color persisted, 50 μL of 30% (w/w) H_2O_2 was added to all cooled tubes, and the samples were heated for another 15 min. Then, the tubes were removed from the heating block and cooled to room temperature. To each tube were added 3.9 mL of deionized water and 0.5 mL of ammonium molybdate (IV) tetrahydrate (2.5% (w/v); cat. no. A1343, Sigma-Aldrich). Samples were mixed with a vortex mixer five to seven times. Then, 0.5 mL of freshly prepared ascorbic acid solution (10% (w/v); cat. no. A5960, Sigma-Aldrich) was added and the samples were mixed again. The tubes were

closed with the screw cap to prevent evaporation and heated at 100°C for 7 min in a water bath. After cooling to ambient temperature, samples were analyzed spectrophotometrically. The wavelength of the spectrophotometer was set to 820 nm, and the instrument was blanked against air before the samples were measured. From the phosphorus standards, a calibration curve was generated, and the amounts of the phosphorus in the test samples were determined. Multiplication of the obtained amount of phosphorus by 0.950 yielded the correct amount of phospholipids. This factor considers the presence of cardiolipin (CL), which contains two phosphate moieties per lipid molecule, in contrast to phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). See Table S2 for more information.

Estimation of the average molecular mass of an *E. coli* phospholipid

For the calculation of the average molecular mass of an *E. coli* phospholipid (766.39 Da), the relative ratio of the three main phospholipids, i.e., PE/PG/CL = 71.39:23.36:5.25 mol/mol %, as well as the average molecular masses of PE (719.302 Da), PG (761.073 Da), and CL (1429.954 Da) (Avanti Polar Lipids), were considered. See Table S2 for more information.

Calculation of the lipid-accessible surface of membrane proteins

The PDB file of the corresponding membrane protein, i.e., UT (3K3F (9)), AdiC (3LRB (10)), and LacY (2V8N (11)), oriented in the lipid bilayer was downloaded from the OPM (Orientations of Proteins in Membranes) database (<http://opm.phar.umich.edu/>) (21). The lipid-accessible surface (\AA^2) was calculated by the Gerstein method (22,23) using a probe radius of 1.88 \AA (approximate radius of a methylene group) (24) and considering atoms from amino acids within the membrane boundaries.

Lipid extraction from SEC peak fractions of purified AdiC in DDM and OM for mass spectrometry

Lipid extraction was performed according to a slightly modified version of the method described by Bligh and Dyer (25). Briefly, 150 μL PBS, 500 μL CH_3OH , and 250 μL CHCl_3 were added to 50 μL of SEC peak fraction ($\sim 0.4 \text{ mg/mL}$ protein concentration) to yield a 1:2:0.8 ratio (PBS/ $\text{CH}_3\text{OH}/\text{CHCl}_3$). The mixture was vortexed for 1 min and centrifuged at $9500 \times g$ for 5 min at 4°C (single-phase extraction). The supernatant was recovered and 250 μL PBS and 250 μL CHCl_3 were added immediately to yield a 1.8:2:2 ratio (PBS/ $\text{CH}_3\text{OH}/\text{CHCl}_3$). The mixture was vortexed again for 1 min and centrifuged ($9500 \times g$ for 5 min at 4°C) (two-phase extraction). The lipids recovered from the lower organic phase were dried under nitrogen and stored at -20°C . The lipid extraction was done one day before electrospray ionization-mass spectrometry (ESI-MS) analysis.

For the direct infusion, samples were dissolved in 1 mL methanol containing 0.2% acetic acid, vortexed for 1 min, and centrifuged ($9500 \times g$ for 5 min at 4°C). The supernatant was transferred to a high-performance liquid chromatography glass vial and analyzed by direct infusion into the ESI-MS.

Mass spectrometry

Equipment

ESI-MS analyses were conducted on an API 4000 QTrap mass spectrometer equipped with a TurboIonSpray probe (AB Sciex, Concord, Ontario, Canada). Data acquisition and analysis were performed using the Analyst software version 1.5.1 (AB Sciex).

MS Detection

The TurboIonSpray probe was operated in negative and positive ionization modes (MS[−] and MS⁺). The source parameters, run at room temperature using nitrogen as a curtain gas and nebulizer, were capillary voltage in positive and negative modes, −4.2 kV and +4.5 kV; curtain gas, 20 psi; GS1, 20 psi; GS2, 0 psi; declustering potential, 70 V; entrance potentials, 10 V; and interface heater ON. Q1/Q3 resolutions were set to −0.7/−0.5 Da and 1/1 Da for both PS and NL in negative and positive modes. The collision energy was set at 40 eV and 50 eV in negative and positive modes, respectively. Collision exit potential was set to 10 V in all experiments. The parameters for tandem MS (MS/MS) experiments on PE and PG in negative and positive modes were precursor ion scan (PS) of −196 (PS−196), neutral loss (NL) of +141 (NL+141), PS of −153 (PS−153), and NL of +171 (NL+171). When charged phospholipids (PLs) lose a diagnostic fragment (neutral or charged) of a defined mass, these fragments can be detected in NL and PS, e.g., neutral fragments of 141 Da for PE and 171 Da for PG in NL. The samples were directly infused using a syringe pump at a speed rate of 10 μ L/min. Each sample was first infused for 3 min to stabilize the signal and the experiments were run consecutively in the order MS[−], PS−169, PS−153, MS⁺, NL+141, and NL+196. The duty cycle was 2 s and the scans were gained during 3 min in multiple-channel acquisition mode. All experiments were acquired in the range 600–900 Da.

MS/MS experiments were performed only in negative mode, using a similar setup as described above except that the mass range recorded was from 100 Da to 10 Da above the molecular mass of the corresponding phospholipid. Experiments were recorded for 2 min.

Model generation of AdiC ternary complexes

The AdiC structure (PDB 3LRB) was placed into a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) bilayer by applying the Membrane Builder Tool of the CHARMM-GUI (<http://www.charmm-gui.org/?doc=input/membrane/>) (26,27). Next, phospholipids were removed manually and only molecules that were in close contact to AdiC were kept. To position detergent molecules appropriately, the former model was used as input file for a PACKMOL run (28). The numbers of phospholipid and detergent molecules arranged around AdiC were according to the experimentally determined values of AdiC purified in DDM and OM.

RESULTS AND DISCUSSION

Determination of the apparent molecular masses of ternary complexes by SEC

The model membrane proteins UT, AdiC, and LacY were purified by metal affinity chromatography in the detergents DDM, UDM, DM, NM, OM, Cymal-7, Cymal-6, Cymal-5, and NG (see Fig. S1 for SDS-PAGE gels). Purified proteins were then submitted to SEC on Superdex 200. Fig. 1 displays representative elution profiles of UT, AdiC, and LacY in the nine different detergents. In contrast to UT and AdiC, which were stable in all detergents, LacY started to aggregate in NM and OM during purification (Fig. 1 C) and could not be purified in NG (no protein yield after affinity chromatography). NM, OM, and NG have relatively short alkyl chains and can be considered as harsh detergents (5). Table 1 summarizes the apparent molecular masses ($M_{app,SEC}$) of UT, AdiC, and LacY in different detergents, as determined by SEC. In all model proteins, the $M_{app,SEC}$ of the ternary complexes decreased with decreasing alkyl

chain length in alkylmaltosides (DDM, UDM, DM, NM, and OM) and with decreasing length of the alkyl chain connecting the cyclohexane and maltoside moieties in Cymals (Cymal-7, Cymal-6, and Cymal-5). It should be noted that a decrease in alkyl chain length implies an increase of the CMC and a decrease of the aggregation number (see Table S3 for detergent properties). Similar trends were observed in SEC experiments with monomeric ADP/ATP carriers from *Saccharomyces cerevisiae* (29,30), corroborating our results.

Determination of the detergent-binding capacities and phospholipid contents of purified UT, AdiC, and LacY proteins

SEC peak fractions were collected for UT, AdiC, and LacY purified in alkylmaltoside and Cymal detergents, and the protein, detergent, and phospholipid contents were determined (see Materials and Methods). Because LacY tended to aggregate in NM and OM, those samples were not considered for detergent and phospholipid determinations. For the glucoside NG, phospholipid contents of UT and AdiC were determined, but not detergent-binding capacities. In contrast to the maltosides, SEC protein peaks overlapped with the empty NG micelles (Fig. S2), making determinations of the detergent content in peak fractions unreliable. LacY in NG was not considered because of aggregation and no protein yields after affinity chromatography. Figs. 2–4, A and B, summarize the determined numbers of bound phospholipids, n_{PL} , and detergent molecules, n_D , respectively, per UT trimer, AdiC dimer, and LacY monomer. Considering Figs. 2 A, 3 A, and 4 A, high CMC detergents had significantly stronger delipidating effects on purified UT, AdiC, and LacY than did low CMC detergents as a direct consequence of the higher detergent concentrations needed when working with high CMC detergents (Table S1). Delipidation was particularly dramatic for monomeric LacY; e.g., with DM, there was only one phospholipid per LacY protein (Fig. 4 A), which of the three model proteins has the smallest hydrophobic membrane-embedded surface (see also Calculation of the hydrophobic lipid-accessible surfaces of UT, AdiC, and LacY, and the correlation with the experimental data). NG and Cymal-5 had exceptional delipidating effects on the model proteins compared to the other detergents tested (Figs. 2 A, 3 A, and 4 A). In summary, the amount of endogenous phospholipid bound to a membrane protein after purification varies strongly with the detergent type and concentration. Delipidation as a function of decreasing detergent chain lengths is more efficient for proteins with smaller hydrophobic membrane-embedded surface. This is nicely illustrated by the abrupt decrease of phospholipids from DDM to UDM in LacY compared to UT and AdiC (Figs. 2 A, 3 A, and 4 A). It should also be considered that an increase of the concentration of the same detergent, e.g., from 0.01% DDM to 0.1% DDM, to

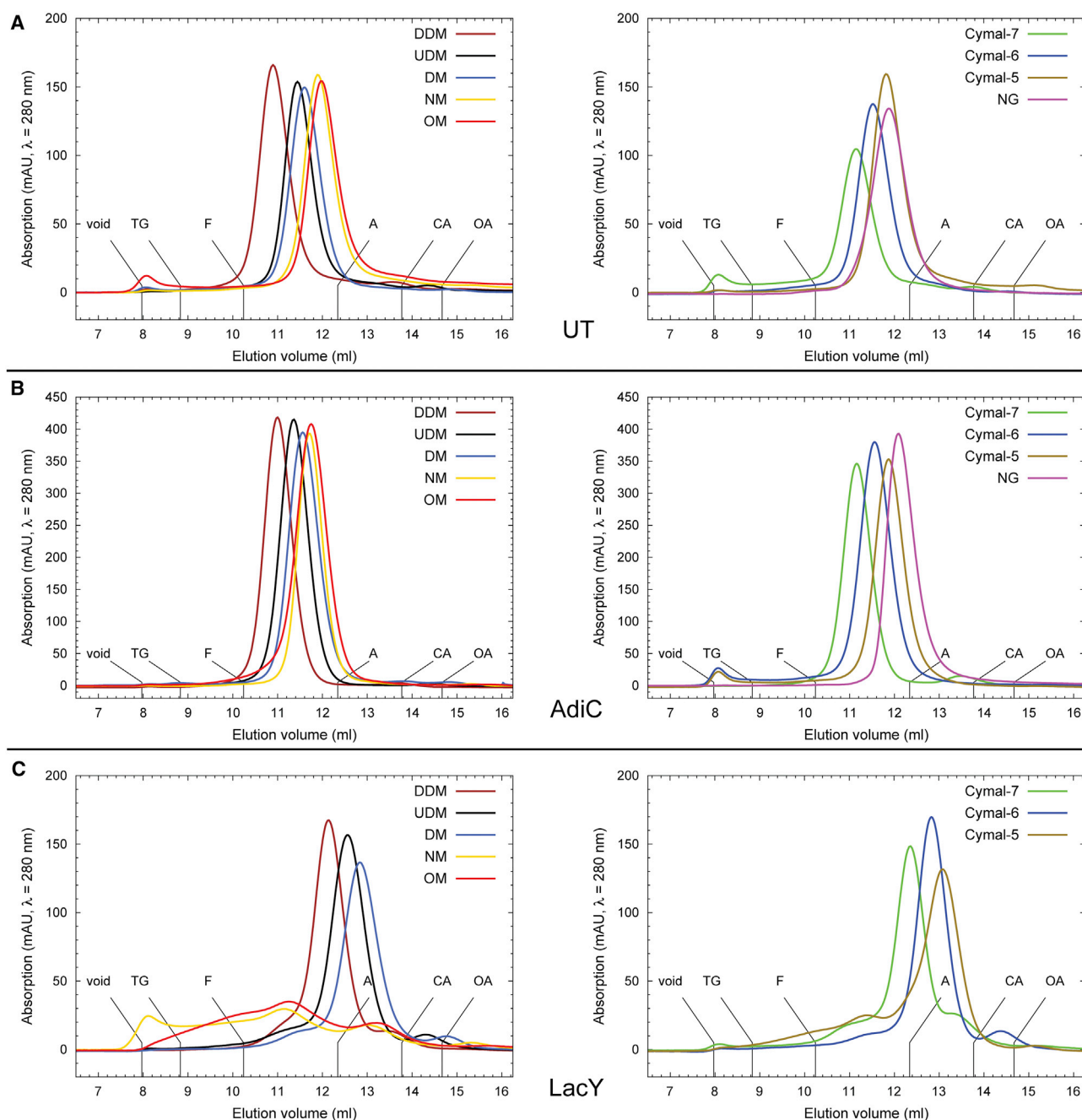


FIGURE 1 SEC analysis of the model membrane proteins UT, AdiC, and LacY purified in nine different detergents. Representative elution profiles of purified UT (A), AdiC (B), and LacY (C) are shown. Alkylmaltosides (DDM, UDM, DM, NM, and OM), Cymals (Cymal-7, Cymal-6, and Cymal-5), and the glucoside NG were used as detergents. UT and AdiC were stable in all detergents. SEC of LacY purified in NM and OM indicated aggregation. LacY was unstable in NG and could not be purified and submitted to SEC. Void volume and retention volumes are indicated for standard proteins thyroglobulin (TG, 669 kDa), ferritin (F, 440 kDa), aldolase (A, 158 kDa), conalbumin (CA, 75 kDa), and ovalbumin (OA, 43 kDa). To see this figure in color, go online.

keep the protein in solution during purification, has substantial delipidating effects on membrane proteins (31). To support this finding, we also purified AdiC using 0.12% instead of 0.04% DDM (i.e., at a threefold higher detergent concentration). This led to a reduction of the copurified phospholipids bound to one AdiC dimer from 52 (0.04%

DDM) to 27 (0.12% DDM) (Fig. 3 A), corroborating the findings of Sigal et al. (31).

The number of detergent molecules bound to a specific, purified membrane protein strongly depended on the detergent used. For maltosides, the number of bound detergent molecules decreased with shorter alkyl chain length. The

TABLE 1 Determination of $M_{app,SEC}$ of ternary complexes by SEC

Detergents	Apparent molecular mass of ternary complex (kDa)		
	UT	AdiC	LacY
DDM	277 ± 4.6	257 ± 4.4	156 ± 10.6
UDM	222 ± 1.5	220 ± 4.9	122 ± 1.7
DM	192 ± 2.2	198 ± 1.9	106 ± 1.3
NM	183 ± 1.8	183 ± 3.0	96 ± 0.3 ^a
OM	174 ± 2.4	179 ± 1.4	87 ± 2.1 ^a
Cymal-7	232 ± 1.6	236 ± 1.3	135 ± 4.1
Cymal-6	203 ± 1.4	205 ± 1.5	110 ± 3.1
Cymal-5	183 ± 0.6	170 ± 3.9	97 ± 1.6
NG	171 ± 0.2	152 ± 2.6	ND

Values represent the mean ± SD from at least two SEC runs with protein from two to five independent purifications. The apparent molecular mass of the ternary complex was determined by SEC. ND (not determined) indicates that the protein aggregated during purification, and SEC could not be performed.

^aThese values are to be considered with caution, since LacY started to aggregate during purification and SEC in these two detergents.

relationship between hydrophobic membrane-embedded surface and detergent binding capacity on the one hand, and the phospholipid content on the other are further discussed below (see Calculation of the hydrophobic lipid-accessible surfaces of UT, AdiC, and LacY, and the correlation with experimental data).

Determination of the phospholipid types bound to purified AdiC in DDM and OM

Of the five alkylmaltoosides tested, AdiC purified in DDM and OM had the highest and lowest amounts of bound endogenous phospholipids (Fig. 3 A). To determine and compare the types of phospholipids bound in these two boundary conditions, we performed ESI-MS/MS analysis of SEC peak fractions of purified AdiC in DDM and OM (Fig. 5).

Phospholipids are charged molecules that show characteristic MS/MS fragmentation patterns. Based on these fragments, different phospholipid classes can be detected in neutral loss scan (NL) and precursor ion scan (PS) experiments; e.g., NL+141 and PS−196 are used for analysis of PEs, and NL+171 and PS−153 for the analysis of PGs (see Materials and Methods) (32,33). In a first step, and as reference, we analyzed *E. coli* polar lipid extract, which only contains PE, PG, and CL, using the method described by Oursel et al. (34). Six PEs and five PGs (Table S4) were identified that corresponded to the main PEs and PGs reported in *E. coli* lipids (34). Their structure was confirmed by MS/MS analysis of the m/z corresponding to $[M-H]^-$ (data not shown). Because CL is a minor constituent of *E. coli* lipids, and because resolution is limited by the use of direct infusion, we were unable to identify CL with our experimental setup. Next, we analyzed *E. coli* total lipid extract and found the six PEs again in all four different

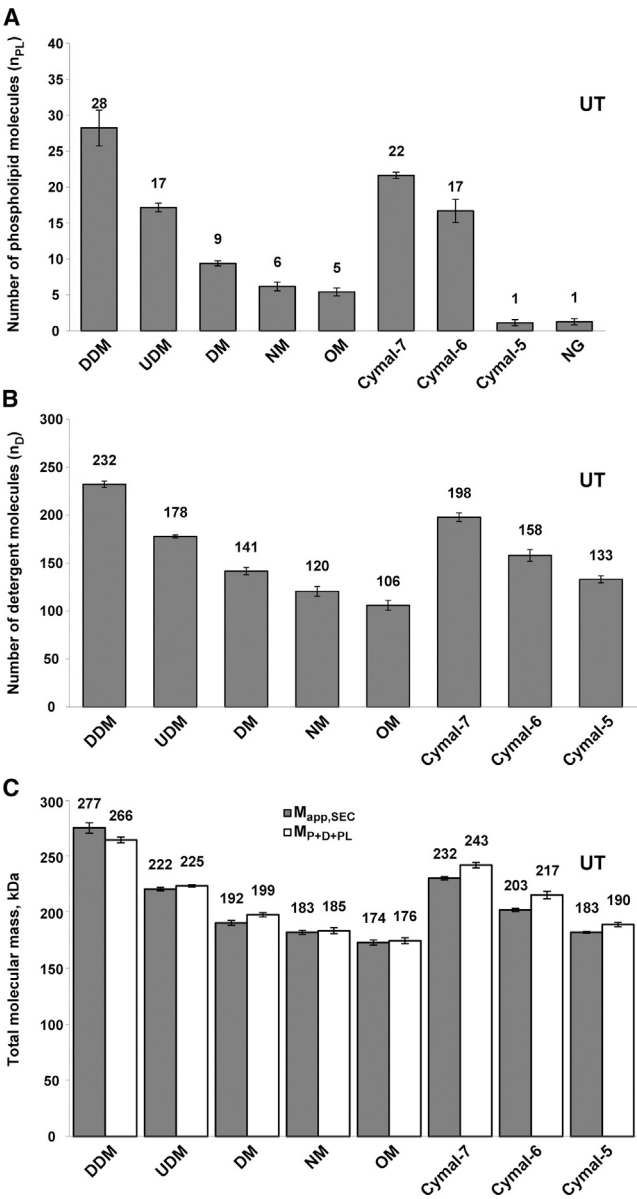


FIGURE 2 (A and B) Determination of phospholipid (A) and detergent molecules (B) bound to purified UT from SEC peak fractions. Alkylmaltoosides (DDM, UDM, DM, NM, and OM), Cymals (Cymal-7, Cymal-6, and Cymal-5), and the glucoside NG were used for purification and SEC. The determined numbers of phospholipid (n_{PL}) and detergent molecules (n_D) bound to UT are indicated. Values represent the mean ± SD from at least triplicates of two to three independent purifications. (C) Comparison of molecular masses of UT ternary complexes in different detergents determined by SEC ($M_{app,SEC}$; Table 1), and protein, phospholipid and detergent determination (M_{P+D+PL}).

experiments (i.e., MS+, MS−, PS−196, and NL+141), whereas the positive mode NL+141 was the most sensitive (Fig. S3, upper). On the other hand, PG detection was in general less sensitive, and we were only able to identify the five PGs using the negative-mode PS−153 (Fig. S4, upper). Therefore, the NL+141 and PS−153 modes were used to determine the types of endogenous phospholipids

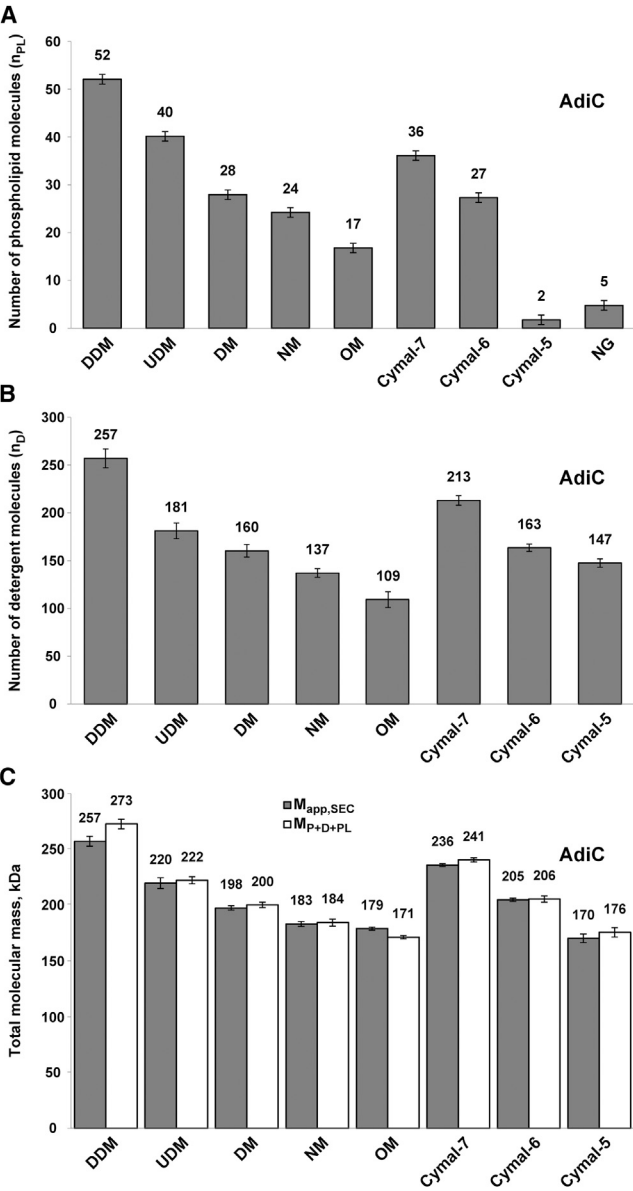


FIGURE 3 (A and B) Determination of phospholipid (A) and detergent molecules (B) bound to purified AdiC from SEC peak fractions. Alkyl-maltosides (DDM, UDM, DM, NM, and OM), Cymals (Cymal-7, Cymal-6, and Cymal-5), and the glucoside NG were used for purification and SEC. The determined numbers of phospholipid (n_{PL}) and detergent molecules (n_D) bound to AdiC are indicated. Values represent the mean \pm SD from at least triplicates of two to three independent purifications. (C) Comparison of molecular masses of AdiC ternary complexes in different detergents determined by SEC ($M_{app,SEC}$; Table 1), and protein, phospholipid and detergent determination (M_{P+D+PL}).

bound to AdiC when purified with DDM and OM (Figs. S3 and S4, middle and lower).

Fig. 5 summarizes the results from ESI-MS/MS analysis of AdiC purified in DDM and OM, showing the identified PEs (Fig. 5 A) and PGs (Fig. 5 B). The relative amounts of bound PE and PG (Fig. 5 C) in AdiC purified in OM were strongly decreased compared to those purified in

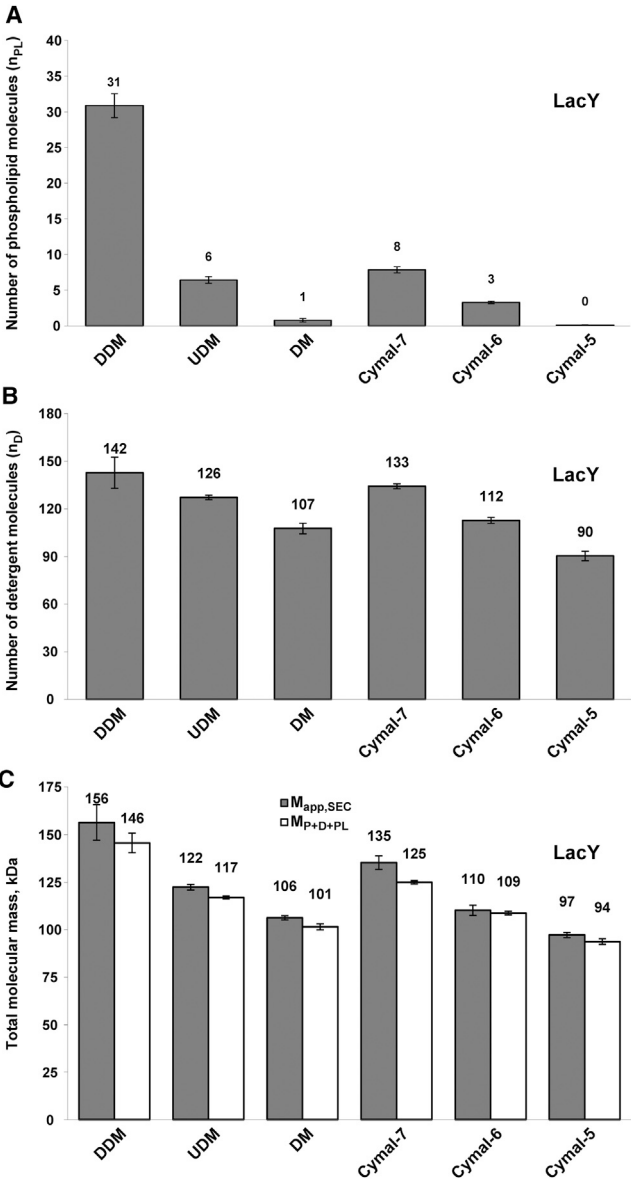


FIGURE 4 (A and B) Determination of phospholipid (A) and detergent molecules (B) bound to purified LacY from SEC peak fractions. Alkyl-maltosides (DDM, UDM, and DM) and Cymals (Cymal-7, Cymal-6, and Cymal-5) were used for purification and SEC. The determined numbers of phospholipid (n_{PL}) and detergent molecules (n_D) bound to LacY are indicated. Values represent the mean \pm SD from at least triplicates of two to three independent purifications. (C) Comparison of molecular masses of LacY ternary complexes in different detergents determined by SEC ($M_{app,SEC}$; Table 1), and protein, phospholipid, and detergent determination (M_{P+D+PL}).

DDM, with the exception of PG-747 in OM (see Table S4 for phospholipid nomenclature). This interesting finding indicated that the negatively charged phospholipid PG-747 is strongly enriched in AdiC samples purified in OM. This enrichment most probably happens during membrane solubilization, where all *E. coli* lipids are still present. In general, the amount of PE in AdiC purified in OM is

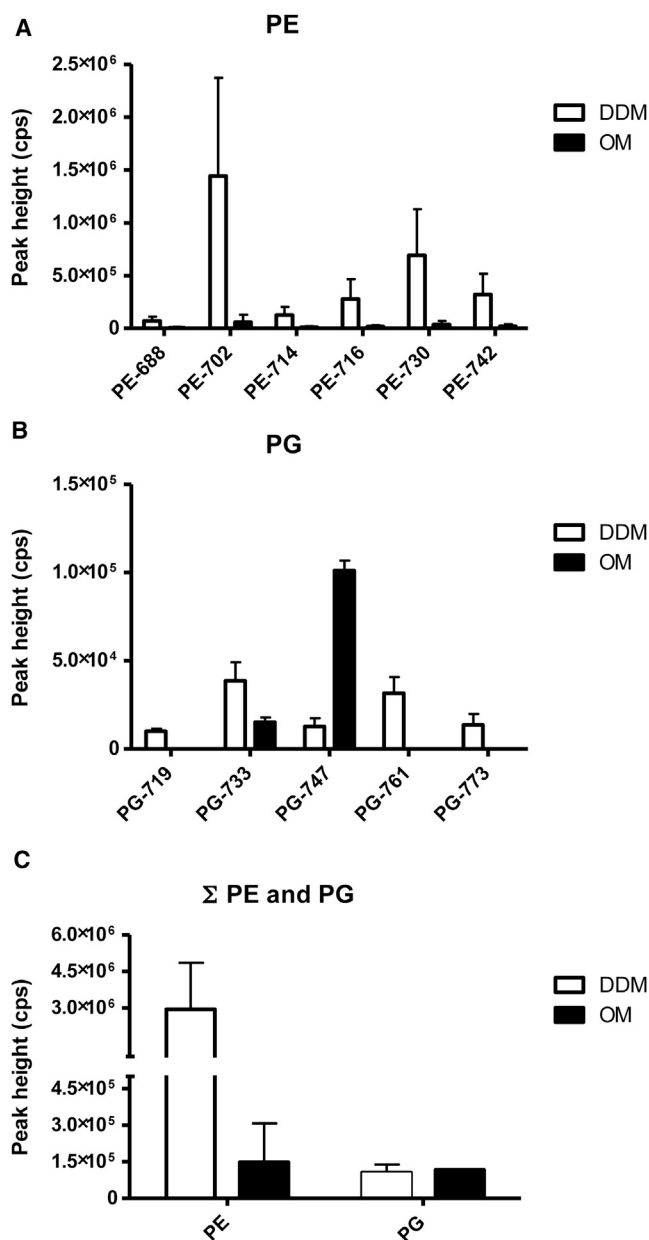


FIGURE 5 Semiquantitative analyses of PEs and PGs present in AdiC purified with DDM and OM. (A and B) Analyses of PEs and PGs using NL+141 (A) and PS-153 (B) modes. (C) Comparison of the relative amounts of PEs and PGs from NL+141 and PS-153 experiments. The sums of all PEs and PGs are displayed. Values represent the mean \pm SD from at least duplicates of two independent purifications. cps, counts/s.

strongly reduced compared to that purified in DDM, whereas the relative amounts of PG stay similar (Fig. 5 C).

Comparison of molecular masses of ternary complexes determined by different methods

Knowledge of the molecular mass, oligomeric state, and amount of bound phospholipid and detergent per model membrane protein allowed calculation of the total molecular

mass of the ternary complexes (M_{P+D+PL}). Calculations were done for UT, AdiC, and LacY and compared with the $M_{app,SEC}$ (Figs. 2 C, 3 C, and 4, C). These comparisons demonstrated that the molecular masses of the UT, AdiC, and LacY ternary complexes determined by SEC ($M_{app,SEC}$) are in general similar to the M_{P+D+PL} values, and thus quite precise and reliable. However, considering that the $M_{app,SEC}$ values for UT and AdiC (Table 1) are comparable, particular care has to be taken when trying to deduce unknown oligomeric states of membrane proteins based on $M_{app,SEC}$ values alone (35).

Calculation of the hydrophobic lipid-accessible surfaces of UT, AdiC, and LacY, and correlation with the experimental data

The atomic structures of UT, AdiC, and LacY were solved (9–11), and the coordinate files are available. Furthermore, the OPM database provides PDB files of these proteins oriented in the lipid bilayer, indicating the membrane boundaries. Using these coordinate files, we calculated the hydrophobic lipid-accessible surfaces of our model membrane proteins as 12,397 Å² for UT, 12,694 Å² for AdiC, and 7,607 Å² for LacY (see Materials and Methods).

Our experimental data allowed the comparison of the phospholipid contents in trimeric (UT), dimeric (AdiC), and monomeric (LacY) membrane proteins. Comparison of UT (Fig. 2 A) with AdiC (Fig. 3 A) indicated that the phospholipid content in these two membrane proteins of different oligomeric states but having similar hydrophobic lipid-accessible surfaces varied significantly, with, for example, 28 and 52 phospholipids in DDM. This significant difference in bound endogenous phospholipids might be attributed to different physicochemical properties of the membrane-embedded surfaces of the two proteins. With the exception of DDM, the numbers of phospholipid molecules bound to LacY were significantly lower than in UT and AdiC. This makes sense, considering that LacY has only ~60% of the hydrophobic membrane-embedded surface of UT and AdiC to accommodate phospholipid molecules. Interestingly, the number of endogenous phospholipid molecules in LacY purified with DDM was high compared to purifications with the other detergents, indicating the mildness of DDM.

As mentioned above, UT and AdiC have similar hydrophobic lipid-accessible surfaces, but retain different amounts of endogenous phospholipids during purification. This makes it possible to estimate whether increased amounts of endogenous phospholipids bound to membrane proteins also significantly increase the size of the detergent micelles. For example, comparable numbers of UDM and OM molecules, i.e., 178 and 181 (UDM), and 106 and 109 (OM), were found in purified UT and AdiC, despite the significant difference in endogenous phospholipids,

i.e., 17 and 40 (UDM), and 5 and 17 (OM) (Figs. 2, A and B, and 3, A and B). Based on these results, important differences in micelle size are not expected within the here determined range of bound endogenous phospholipids.

Specific amounts of detergent bound to membrane proteins are needed to keep them in solution. These detergent molecules are sequestered during the solubilization step, where isolated membranes are exposed to high detergent concentrations. Table S5 displays the amount of detergent bound to purified UT, AdiC, and LacY in terms of empty detergent micelles (see Table S3 for aggregation numbers of detergents, which define empty micelles). It is clear that the larger the hydrophobic lipid-accessible surface of the protein, the higher is the empty detergent micelle/protein (complex) ratio; e.g., 1.6, 2.0, and 2.3 empty DM micelles are needed to keep LacY, UT, and AdiC solubilized. It should be noted that dimeric AdiC has a larger hydrophobic lipid-accessible surface than trimeric UT (Table S5) despite its lower oligomeric state. Therefore, the hydrophobic lipid-accessible surface area is critical for the amount of detergent bound and is not necessarily an indicator of the oligomeric state of the membrane protein.

Finally, we evaluated whether the determined detergent binding capacities and phospholipid contents make sense and can be accommodated around the hydrophobic membrane-embedded surface of a membrane protein. To this aim, we modeled the structure of AdiC in terms of the numbers of phospholipid and detergent molecules determined at the two extremes in the alkylmaltoside series (Fig. 6). From this model, it is evident that the determined numbers of detergent and phospholipid molecules make sense and can be accommodated around the hydrophobic membrane-embedded surface. Furthermore, the models support the experimental results (Figs. 2, A and B, 3, A and B, and 4, A and B) showing that the size of the detergent and lipid belt surrounding the hydrophobic lipid-accessible surface of purified membrane proteins can be tuned by the appropriate choice of detergent.

CONCLUSION

Analyses of the amounts of detergent and endogenous phospholipid molecules bound to purified membrane proteins have been neglected in the past. Consequently, information on the composition of such ternary complexes is very sparse. We have conducted a systematic study and characterized ternary complexes of three model membrane proteins purified in nine different detergents and determined their molecular masses, detergent binding capacities, and phospholipid contents. From the experimental results, the insights gained were tentatively put into a general context as follows. 1), The number of detergent molecules bound to purified membrane proteins is dependent on the physicochemical properties of the detergents used and the hydro-

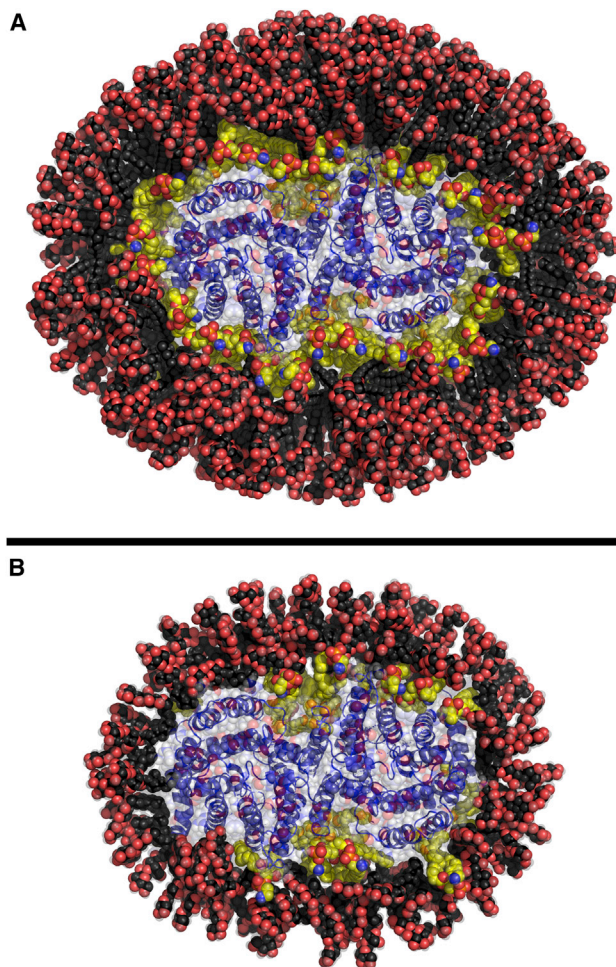


FIGURE 6 (A and B) Ternary complex models of AdiC purified in DDM (A) and OM (B). The ternary complexes shown contain the AdiC-dimer protein (blue cartoon and transparent spheres), phospholipid (yellow spheres), and detergent molecules (black spheres). Oxygen and nitrogen atoms are colored red and blue, respectively. The ternary complexes in A and B are composed of 257 DDM and 52 POPE, and 109 OM and 17 POPE molecules, respectively. These numbers for phospholipid and detergent molecules used for model building are based on the experimentally determined values (Fig. 3). Images were prepared with PyMOL software (www.pymol.org). To see this figure in color, go online.

phobic lipid-accessible protein surfaces, i.e., the size of the surface area to which the hydrophobic detergent tail can bind. 2), The number of endogenous phospholipid molecules bound to purified membrane proteins is dependent on the hydrophobic lipid-accessible protein surfaces, as well as the detergent types and concentrations used for purification. 3), As reflected by our experimental data (Figs. 2–4 and 6), the size of the detergent and lipid belt surrounding the hydrophobic lipid-accessible surface of purified membrane proteins can be tuned by the appropriate choice of detergent. 4), Exceptional delipidating effects on membrane proteins were found for the detergents NG and Cymal-5 (see recent structure reports using NG and Cymal-5 (36–40)). In agreement, controlled delipidation together with a minimally

sized detergent/lipid belt have been shown to be important in allowing crystal contact formation and growth of highly ordered membrane protein 3D crystals for structure determination (12,13). 5), As illustrated by AdiC purified with two detergents with very different physicochemical properties (i.e., DDM and OM), the types of endogenous phospholipids bound to purified membrane proteins can significantly vary depending on the detergent used. 6), Furthermore, we show that SEC is a suitable method to determine the molecular masses of ternary complexes, which might be successfully applied to other membrane proteins with structures similar to those of UT, AdiC, and LacY.

SUPPORTING MATERIAL

Four figures, five tables, and reference (41) are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(14\)00261-6](http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00261-6).

Financial support from the University of Bern, the Swiss National Science Foundation (grant 31003A_144168 to D.F.), and the National Centre of Competence in Research (NCCR) TransCure (to D.F. and J.G.) is gratefully acknowledged.

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